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Alopecia areata is the second most common form of hair loss in humans after androgenetic alopecia. Although a variety of animal models for alopecia areata have been described, currently the C3H/HeJ mouse model is the most commonly used and accepted. Spontaneous hair loss occurs in 15%-25% of older mice in which the lesions wax and wane, similar to the human disease, with alopecia being more common and severe in female mice. Full-thickness skin grafts from mice with spontaneous alopecia areata to young, normal-haired, histocompatible mice provide a highly reproducible model with progressive lesions that makes it useful for drug efficacy and mechanism-based studies. As alopecia areata is a cell-mediated autoimmune disease, transfer of cultured lymph node cells from affected mice to unaffected, histocompatible recipients also promotes disease development and provides an alternative, nonsurgical protocol. Protocols are presented to produce these models such that they can be used to study alopecia areata and to develop novel drug therapies.

Basic Protocol 1: Full-thickness skin grafts to reproducibly induce alopecia areata in C3H/HeJ mice
Basic Protocol 2: Adoptive transfer of cultured lymphoid cells provides a non-surgical method to induce alopecia areata in C3H/HeJ mice

Keywords: alopecia areata • cell transplants • drug efficacy trials • full-thickness skin grafts • mouse models

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INTRODUCTION

Biological Process Under Study

Alopecia areata (AA) is a common, non-scarring inflammatory hair disorder that affects men, women, and children. The condition is characterized by spontaneous hair loss that may be followed by periods of remission, recurrence, and exacerbation (Alkhalifah et al.,

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2010a). AA in humans initially manifests as patchy hair loss during its acute phase, which may, over time, escalate to complete scalp hair loss (termed alopecia totalis) or total body hair loss (referred to as alopecia universalis) in some individuals (Alkhalifah et al., 2010a). A recent study estimated the lifetime prevalence of AA in North America to be around 2.1% (Mirzoyev et al., 2014). This condition represents approximately 25% of cases of hair loss encountered in dermatology clinics (Kos & Conlon, 2009; McMichael et al., 2007). The course of AA is unpredictable and, despite recent progress with new treatment developments, no existing therapy is definitively curative or preventive (Alkhalifah et al., 2010b). The unpredictable nature of the disease often leads to significant emotional distress, with many patients experiencing depression and anxiety (Wang & McElwee, 2011).

Throughout its history, the association between AA and inflammation has been well established (Broadley & McElwee, 2020). Although AA can manifest in various clinical and etiological forms (Gong et al., 2020), it consistently presents as a non-scarring form of hair loss (Bertolini et al., 2020). Histological analysis reveals that anagen-stage hair follicles are surrounded and infiltrated by inflammatory cells, predominantly $CD8^+$ and $CD4^+$ T cells (Perret et al., 1984), along with antigen-presenting cells (APCs) (Fuentes-Duculan et al., 2016; Ito et al., 2020), mast cells (Bertolini et al., 2014), natural killer (NK) cells (Ito et al., 2008), and in some cases eosinophils (Zhang et al., 2013). Notably, $CD8^+$ T cells are often found within follicular structures, whereas $CD4^+$ T cells are more commonly located around the periphery of follicles (Perret et al., 1984; Ranki et al., 1984; Zoller et al., 2002). Recent advances in both basic and clinical immunology have determined that AA is likely driven by a Th1-type autoimmune response mediated by $CD8^+$ T cells targeting hair follicles (McElwee et al., 2013).

AA is not unique to humans; it is not even specific to mammals. Non-scarring focal inflammatory alopecias have been observed sporadically occurring in species from dogs and cats to horses and cattle (Colombo et al., 2004; Hoolahan et al., 2013; McElwee, Boggess, Olivry, et al., 1998; Scarampella & Roccabianca, 2018; Timm et al., 2010; Tobin et al., 1998). Beyond mammals, isolated studies indicate the avian world is not immune to the development of AA-like features (Smyth & McNeil, 1999). This cross-species prevalence underscores the fundamental biological mechanisms underlying AA and highlights the potential for the development of diverse animal models for use in AA in research. Over time, several disease models have been put forward as potential tools in the study of AA pathogenesis and the advancement of treatments (Sundberg et al., 2015). Below we describe protocols for the development and maintenance of what is currently the best-known and most widely used rodent model for AA, the C3H/HeJ inbred mouse model.

Knowledge Gaps in AA

In the realm of AA research, key knowledge gaps persist, posing challenges to understanding disease pathogenesis and to the development of new and more effective therapies. The precise immunological mechanisms underpinning the initiation and perpetuation of the follicular attack remain incompletely understood. Although T-cell-mediated autoimmunity is a central feature, the specific triggers and the regulatory pathways involved in this process need further elucidation. The roles of various immune cells in the pathogenesis of AA, including dendritic cells, natural killer cells, mast cells, and macrophages, are not fully defined. Of equal significance, the specific targets of the immune response against anagen-stage hair follicles remain to be comprehensively identified. Although limited evidence points towards hair-follicle-expressed peptides derived from keratinocytes and/or melanocytes as immune targets, the primary initiating peptides, and how they are presented and activate the immune system, are unknown.

There is also a lack of clarity regarding the genetic basis of the disease. Despite the identification of numerous susceptibility loci through rodent genetic mapping (Sundberg et al., 2003; Sundberg et al., 2004) and large-scale human genome-wide association studies (GWAS; Petukhova et al., 2010), the functional implications of these genetic variants in AA disease pathogenesis are poorly understood. This gap in understanding blocks the development of genetically targeted therapies. Clinical observations suggest that, even with a genotype that predisposes an individual to AA, there must be a trigger for the overt onset of disease to occur. Environmental factors that contribute to the onset and exacerbation of AA are inadequately characterized. This includes a limited understanding of how lifestyle, diet, stress, allergens, toxins, and other external factors might interact with genetic predispositions to initiate and/or exacerbate the condition (Wang & McElwee, 2011).

Beyond comprehending AA disease pathogenesis, there is a significant need for more robust and standardized methodologies to evaluate the potential efficacy and safety of new therapies. Most new treatments are initially studied using animal models before progressing to clinical trials. There are undoubtedly limitations to the use of rodent models for investigating human diseases and developing new treatments. However, the heterogeneity of human populations, the difficulty and cost in accessing large numbers, and the ethical limitations of human research necessitate the use of *in vitro* and *in vivo* models. Mouse models can address many of the knowledge gaps and enable advancement in our understanding and management of AA.

Limitations of Current AA Model Methods

There are very few well-validated models of AA. *In vitro* models have been developed to investigate how immune cells might target hair follicles (Fehrholz & Bertolini, 2020), but AA involves immune activity beyond the skin, with fate decisions as to the development of AA and response to treatment being made in skin-draining lymph nodes, spleen, and probably thymus. Recapitulating body-wide immune system activity *in vitro* is not currently possible. *In silico* modeling of AA may be possible in the future, but currently our understanding of the disease is too limited to begin developing comprehensive models, beyond genetic evaluation (Abd El-Raheem et al., 2020). For the foreseeable future, *in vivo* rodent models are expected to continue to be the predominant tools for advancing our understanding of AA (Sundberg, Berndt, Sundberg, et al., 2011).

AA is a complex polygenic disease (Petukhova et al., 2010; Sundberg et al., 2003; Sundberg et al., 2004), with moderate phenotype penetrance, in rodents and humans and probably all other affected species as well. Therefore, it can be very difficult to obtain mice, or animals of any other species, with this disease in numbers practical for use in research. Some AA model vendors will screen production colonies for affected mice and sell them at a premium, but this service has become less readily available in recent years. Obtaining affected mice from other investigators using these models is one possible resource, though supply tends to be restricted. Active research colonies are still relatively few and currently limited to China, Israel, Japan, South Korea, the UK, and the United States (personal observations). Several inbred mouse strains are prone to spontaneously developing AA (McElwee et al., 1999; Sundberg et al., 2015), but disease expression is infrequent, and the models have limited validation in the published research literature. The most commonly used animal model for AA research, and the best validated, is derived from the C3H/HeJ inbred mouse strain.

Advantages of the Techniques Described

Using a colony of mice to study AA confers several advantages: Not least, as the C3H/HeJ mouse model is fully inbred, the genetic background on which the AA phenotype is expressed is known (sequenced and annotated; GenomeMUSter, Mouse

Phenome Database, *https://phenome.jax.org/genotypes*), and the C3H/HeJ mouse genome can be specifically manipulated via transgenic and targeted mutations. Further, using mouse models allows researchers to control environmental variables more tightly, which is particularly important in investigating and understanding how these factors may contribute to disease onset and progression. Compared to spontaneous models, in which the onset of disease may be unpredictable and the time course protracted, induced models allow a more rapid induction of AA. This accelerates the research timeline and provides larger numbers of mice, enabling quicker generation of data.

Using C3H/HeJ mice in which AA is induced through skin grafts or immune cell injections, several additional advantages emerge, particularly from a research perspective: In induced models, the timing of disease onset and progression can be precisely controlled. This is helpful for studying the early stages of AA, understanding the sequence of immunological events in disease pathogenesis, and evaluating the efficacy of therapeutic interventions at specific disease stages. Also, induced models tend to exhibit more uniform patterns of disease development, which is beneficial for reproducibility and consistency in experimental outcomes. This uniformity is particularly advantageous when comparing the efficacy of different treatments or studying the disease's pathophysiology.

Types of Data that can be Obtained

Mouse models have enabled, and will continue to enable, the collection of data pivotal for understanding the complex immunological underpinnings of AA. The C3H/HeJ mouse model of AA has been compared against human AA, and although there are some differences in the number and location of immune cells infiltrating alopecia lesions, overall the mouse model is comparable to the human disease (McElwee, Freyschmidt-Paul, Sundberg, et al., 2003; McElwee, Yu, Park, et al., 2005; Pratt et al., 2017; Suzuki et al., 2016). Through the controlled induction of AA, early-stage pathogenic events can be studied, even before the onset of visible hair loss (McElwee, Silva, Boggess, et al., 2003). This feature of the AA mouse model will likely be critical in deciphering the sequence of immunological events that trigger the disease, something that is not possible to do with humans. In addition, the uniformity of disease progression in these models is important for experimental consistency, especially when assessing the efficacy of novel therapeutics or dissecting the pathophysiological mechanisms of AA onset and progression. It is relatively easy to use the skin graft or cell transfer protocols to produce large numbers of mice with AA that can be used in multiple cohorts to compare drug treatments and/or dosages.

Furthermore, these models allow exploration of the roles of specific immune cells and pathways in AA's pathogenesis. Induced AA models offer the flexibility to customize the disease model according to specific research needs. For example, by transferring specific immune cell populations and combinations thereof, researchers can dissect the roles of particular cell types or pathways in disease pathogenesis (McElwee, Freyschmidt-Paul, Hoffmann, et al., 2005). This may be invaluable for understanding the complex immune mechanisms underlying AA. Researchers could also introduce immune cells with genetic modifications or use skin grafts from genetically altered mice, or grafted to histocompatible mutant mouse strains, to study the roles of specific genes or proteins in the disease process (Freyschmidt-Paul et al., 2002; Freyschmidt-Paul et al., 2003; Freyschmidt-Paul et al., 2006).

Implications/Relevance for Human AA

The insights gleaned from mouse AA models are highly relevant to understanding the human disease. The data obtained from these models can be used to elucidate the genetic and immunological landscape of human AA. This is particularly significant given the polygenic nature of AA and its moderate phenotypic penetrance observed across species.

By exploring the disease in a controlled setting, these models provide a foundation for developing targeted therapies and personalized treatment approaches for human AA. Additionally, the standardization of protocols in mouse models sets a benchmark for clinical research, offering a preclinical data template in preparation for human trials. This is important in the context of the current limitations with human-centric research and trials, particularly due to population heterogeneity, ethical constraints, and regulatory authority expectations.

The translation of findings from mouse models to human AA has the potential to transform treatment strategies, moving towards more effective, tailored, and preventive therapeutic interventions. For example, the highly reproducible skin graft AA mouse model (McElwee, Boggess, King, et al., 1998) has been used to study the mechanisms of disease (Carroll et al., 2002; McElwee, Boggess, Burgett, et al., 1998; Pratt et al., 2017; Sundberg, McElwee, Carroll, et al., 2011) and to test treatment strategies (Sun et al., 2008), ultimately leading to the first successfully approved drug to treat the human disease (Dai et al., 2021; King et al., 2022; Wang et al., 2018; Xing et al., 2014). In summary, mouse models, particularly the well-validated C3H/HeJ strain, serve as a critical bridge in AA research that enhances our understanding of the disease's pathogenesis and informs the development of more effective treatments for human AA.

Description of Protocols

Here, we describe the essential protocols for producing the C3H/HeJ mouse AA model and assessing the disease phenotype. Basic Protocol 1 describes the process of grafting skin taken from spontaneous AA affected mice onto naïve recipients to transfer the disease. This technique has been used for many years and is well-validated in published literature. Basic Protocol 2 involves transferring AA to naïve hosts by injecting them with skin-draining lymph node cells taken from spontaneous-AA-affected mice. This more recent development avoids the need for surgical facilities and associated expertise.

STRATEGIC PLANNING

One approach to AA research using a disease model is to develop a colony of AA affected mice "in house" so that they are available as donors and recipients for the protocols described below. AA naturally occurs in 15-25% of C3H/HeJ mice by ∼1 year of age more. Disease onset is often associated with loss of fecundity, the time at which the mice stop breeding. As with humans, naturally occurring AA in mice waxes and wanes, resolves or expands (Sundberg, Cordy, & King, 1994), making the natural disease a difficult model to utilize for investigating mechanisms of disease progression, response to therapy, etc. However, in many cases AA lesions will eventually progress to involve the entire animal's skin: the mouse equivalent of alopecia universalis. Of note, whereas all pelage hair types can be affected in mouse AA, vibrissae remain largely unaffected regardless of hair loss extent or time duration of AA lesions.

Females are more frequently and more severely affected by AA, so most research only uses C3H/HeJ female cohorts. This also has the added advantage that they do not fight as much as male mice, which lessens the chance of skin graft damage (see below). Most spontaneous AA cases develop in mice ∼9-12 months of age, although mice can occasionally be affected beginning as young as 4 months (unpublished observations). Most commercial production colonies retire their breeders at ∼5 months for C3H/HeJ mice because females tend to develop a high frequency of ovarian cysts and tumors that shortens their reproductive life (Husler et al., 1998). As such, mice with clinical features of spontaneous AA are rare even in large production colonies. Therefore, establishing a colony of AA-affected mice can become very time consuming; however, it is critical to be able to produce adequate numbers of affected mice for investigations.

Groups of 20 or more retired breeder female C3H/HeJ mice can be obtained from The Jackson Laboratory, Bar Harbor, ME. Alternatively, research investigators may be willing to provide a limited number of affected mice. Unaffected C3H/HeJ retired breeders, ~5 months of age, can be obtained in large numbers for creating a colony. Some will develop AA and can be used as donor mice for skin graft or cell transfer promotion of AA. C3H/HeN mouse colonies in Asia have been used in research publications and apparently do spontaneously develop AA (Ito et al., 2020; Zheng et al., 2024). Isolated examples of spontaneous AA have also be observed in U.S. colonies of C3H/HeN and C3H/OuJ mice, which are histocompatible with the C3H/HeJ strain (McElwee et al., 1999), though the specific parameters of spontaneous AA in these colonies have not been studied.

Once C3H/HeJ retired females are obtained, it may still take several months to obtain clinically affected AA mice to use as skin graft or cell donor animals for the protocols described below. Nothing can be done until you have affected mice. As such, this takes preplanning, mouse room space, regular monitoring of the mouse colony, and several months before mice with AA are available for research use. Having large cages that can house multiple mice together, combined with having mostly females, which fight less than males, enables one to maintain several boxes to provide a source of affected mice that can be used as needed.

C3H/HeJ mice are not always easy to breed, and some laboratories have been unsuccessful in building their own mouse colony. In part, these problems have been traced to a high-stress animal house environment, including poor handling techniques, as well as issues such as air filtration systems producing high-pitched sounds beyond human hearing (unpublished observations). Older females have a strain-specific predilection for ovarian dysfunction (Husler et al., 1998), which can also complicate maintenance of a C3H/HeJ mouse colony. The cost of maintaining a colony should also be considered in comparison to supply from commercial colonies. Standard colony-maintenance protocols are available elsewhere (*https://www.jax.org/jax-mice-and-services/colony-management*).

NOTE: All protocols involving animals must be reviewed and approved by the appropriate Animal Care and Use Committee and must follow regulations for the care and use of laboratory animals.

BASIC PROTOCOL 1

FULL-THICKNESS SKIN GRAFTS TO REPRODUCIBLY INDUCE ALOPECIA AREATA IN C3H/HEJ MICE

C3H/HeJ mice can develop a spontaneous, complex polygenic, AA-like hair loss (Sundberg et al., 2003; Sundberg et al., 2004) that undergoes stages of waxing and waning (Sundberg, Cordy, & King, 1994), such that clinically evident areas of alopecia vary greatly between subjects and over time. This complicates the use of spontaneous AA models as drug-screening tools. Full-thickness skin grafts are used to investigate the role of inflammation in many skin disease models (Sundberg & Rice, 2023) to determine whether the inflammation observed histologically is driving the skin lesions or whether hair follicle dystrophy results in localized, chronic inflammation as a sequela (McElwee, Boggess, King, et al., 1998; Sundberg, Dunstan, et al., 1994). Step-by-step methods for the surgical full-thickness skin graft procedure are described below.

Materials

Female C3H/HeJ donor mice with at least 70% hair loss (see Strategic Planning, above)

8-week-old female graft recipient mice: e.g., Jackson Laboratory strain no. 000659 (The Jackson Laboratory, Bar Harbor, ME; *http://jaxmice.jax.org/*)

Iodine surgical solution (Betadine Antiseptic Prep Solution, Blowout Medical, *https://www.blowoutmedical.com/*; Purdue Pharma, Stamford, CT, cat. no. 6761815017)

70% (v/v) ethanol

0.9% saline solution, sterile (Vet One, Boise, ID, cat. no. 510224), room temperature

Anesthesia: isoflurane (MilliporeSigma, Burlington, MA, cat. no. 792632) or 2,2,2-tribromoethanol (MilliporeSigma, cat. no. T48402)

Carprofen (Rimadyl, Pfizer Animal Health, New York, NY)

Lubricating ointment (Puralube® Ophthalmic Ointment, Pharmaderm Animal Health, Melville, NY, cat. no. 11897)

Surgical glue (Surgi-lock 2oc, Meridian Animal Health, Omaha, NE, cat. no. 78657941)

Bupivacaine solution: MARCAINE 0.5% 50CC MDV Marcaine Bupivacaine HCl Injection 5 mg/ml, 50 ml, MWI Animal Health, Boise, ID, cat. no. MWI sku 029545), diluted to 0.1% with sterile phosphate-buffered saline (PBS)

Sulfamethoxazole (Sulfamethoxazole+tmp, oral susp 200-40 mg/5 ml 473 ml by Aurobindo, Cherry Flavor, McKesson Corporation, Irving, TX, cat. no. 825443)

Euthanasia method (CO_2 or other method with appropriate Institutional Animal Care and Use Committee (IACUC) or appropriate institutional approval)

Electric clippers (Oster Finisher Trimmer, Oster Professional Products, Newell Brands, Atlanta, GA USA, cat. no. 76059-030)

Surgical instruments, sterile (Roboz Surgical Instrument, Gaithersburg, MD):

- Graefe forceps, 4 in. (10 cm) long, serrated, slight curve, 0.8-mm tip, cat. no. RS-5135
- Micro dissecting scissors, straight, sharp, 3.5 in., cat. no. RS-5910
- Micro Dissecting scissors curved sharp, 4.5 in., cat. no. RS-5913
- Baby Derf needle holder, 4.25 in., cat. no. EA-7820
- Ear punch, cat. no. 65-9900
- Micro dissecting serrated, slight curve forceps, 4 in., cat. no. RS-5135

Tissue culture dishes, sterile: e.g., 35 × 10-mm petri dishes (Fisher, Hampton, NH)

Surgical tape: 3M™ Micropore™ Surgical Tape 1530-1, 1 in. (3M Medical Surgical Division, St. Paul, MN) or 3M™ Steri-Strip™ Adhesive Skin Closures (3M Canada Corporate Headquarters, London, ON, Canada)

Surgical drape (transparent rodent drape, 8 × 8 in. with 2 × 2-foot opening; Steris, Mentor, OH, cat. no. 0808CPADST)

Sutures, sterile: 5-0 Dexon S (Butler Schein, Dublin, OH, cat. no. J385H) or vicryl 5-0 (Ethicon, Raritan, NJ, cat. no. J493G)

Nonadhering dressing pad, sterile (Tefla pad; Kendall, Boston, MA, cat. no. KEN1961)

Self-adhesive bandage (Coban, 3M Medical Surgical Division, St. Paul, MN)

Wound clips (Stoelting, Wood Dale, IL)

Autoclip applier (Stoelting, cat. no. 59043)

Autoclip remover (Stoelting, cat. no. 59046)

Autoclips, box of 1000 (Stoelting, cat. no. 59047)

Mouse acclimation

All procedures must be done with the approval of The Institutional Animal Care and Use Committee or other appropriate regulatory committee for the location where the work will be done.

1. Acclimate the mice to the new facilities (for ~2 weeks) if necessary, and use mice at a minimum of 8 weeks of age.

   Typically, 10-12 weeks of age is the preferred age for grafting. Mice need to be maintained under carefully controlled husbandry conditions, with particular attention to diet (see Critical Parameters).

Figure 1 Removal of donor skin. After euthanasia, alopecic skin is excised using aseptic surgical methods in 1-cm-diameter circular units. As many as possible are taken, transferred to sterile culture dishes, and then grafted onto recipient mice.

Preparation of donor grafts

2. Euthanize donor mice with extensive AA according to the recommendations of the American Veterinary Medical Association (Leary et al., 2020).

   Ideally, representative areas should be biopsied to confirm the diagnosis, as other diseases can cause alopecia in mice (Sundberg et al., 2022).

3. Shave the dorsal skin of euthanized mice with electric clippers. Remove any remaining cut hairs (stubble) using adhesive tape.

4. Disinfect the skin using a routine surgical preparatory method with surgical iodine followed by 70% ethanol.

   Solutions are applied to the center of the surgical field and then worked centrifugally to the periphery. This procedure is done initially for harvesting grafts on the dorsal surface, and the process is then repeated before harvesting ventral skin.

5. Remove multiple 1-cm donor grafts (Fig. 1). Elevate a fold of skin from the desired site using sterile forceps. Excise a circular or oval piece of skin using curved, sharp micro-dissecting scissors. Harvest grafts from both the ventral and dorsal surfaces of the donor mice.

   Whether the grafts are from dystrophic anagen or telogen donor skin makes no apparent difference to the success of AA grafting (unpublished observations).

6. Place the excised grafts, epidermal side down, on a sterile flat surface, such as a tissue culture dish.

7. Scrape away any subcutaneous fascia using forceps.

8. Transfer the donor grafts to another tissue culture dish containing sterile 0.9% saline solution at room temperature, dermal side down.

9. Store grafts for up to 4 hr at room temperature until ready to graft them onto recipient mice.

Surgical procedure for graft recipients

10. Anesthetize graft recipient mice using an intraperitoneal injection of tribromoethanol (0.2 ml per 10 g body weight; *https://phenome.jax.org/projects/Johnson4/protocol*; Boggess et al., 2006).

    Alternatively, isoflurane gas anesthesia can be used (https://www.jax.org/-/media/jaxweb/files/research-and-faculty/tools-and-resources/aging-center-protocols/rtp_awc_062-isoflurane-anesthesia-in-rodents.pdf).

11. Inject carprofen subcutaneously (0.1 ml per 10 g body weight) to alleviate pain or distress immediately after the administration of tribromoethanol.

12. Apply a lubricating ointment, such as Puralube, to the eyes to prevent drying.

13. Surgically prepare the dorsal skin surface at the level of the thoracolumbar junction, as done for the donor skin sites.

14. Position the anesthetized recipient mice in ventral recumbency to provide easy access to the graft site (Fig. 2A).

15. Cover the dorsal area using a sterile clear plastic drape (Fig. 2B) with a hole cut in it to expose the surgical site.

16. Elevate the skin at the graft site to create a fold using forceps.

17. Excise a circular or oval piece of skin (1 cm or slightly less in diameter to match the donor skin graft) using curved scissors (Fig. 2C).

18. Place the donor graft in the recipient site (Fig. 2D).

19. Orient the graft 180° from normal to allow the hair to grow in the opposite direction to the recipient's hair, for easy identification of the graft site after healing (McElwee, Boggess, King, et al., 1998).

20. Use four simple interrupted sutures (5-0 Dexon S or similar material) on opposite sides of the graft to fix the skin graft in place (Fig. 2E).

21. Apply four spots of surgical glue (Surgi-lock 2oc) to the edges of the graft in between the sutures (Fig. 2F and 2G).

    Some institutions may not allow this due to concerns that the glue might get between the graft and host skin; as an alternative, additional sutures can be used.

22. Drip a 0.1% solution of bupivacaine topically onto the graft site after the graft is secured in position. Cover the graft site with a sterile nonstick dressing pad (Tefla pad; Fig. 2H), and then secure that in place with surgical tape and cover it with self-adhesive bandage (Coban) to create a pressure bandage (Fig. 2I).

    Care should be taken when wrapping the mouse with the self-adhesive bandage to avoid compromising normal respiration and locomotion.

23. Place wound clips in the skin at the back of the neck and thorax, and on each side of the pelvis, to hold the bandages in place to make it difficult for the mouse to remove the bandage (Fig. 2I).

    Some institutional regulatory committees will not allow wound clip use; as an alternative, bandages can be sutured into place.

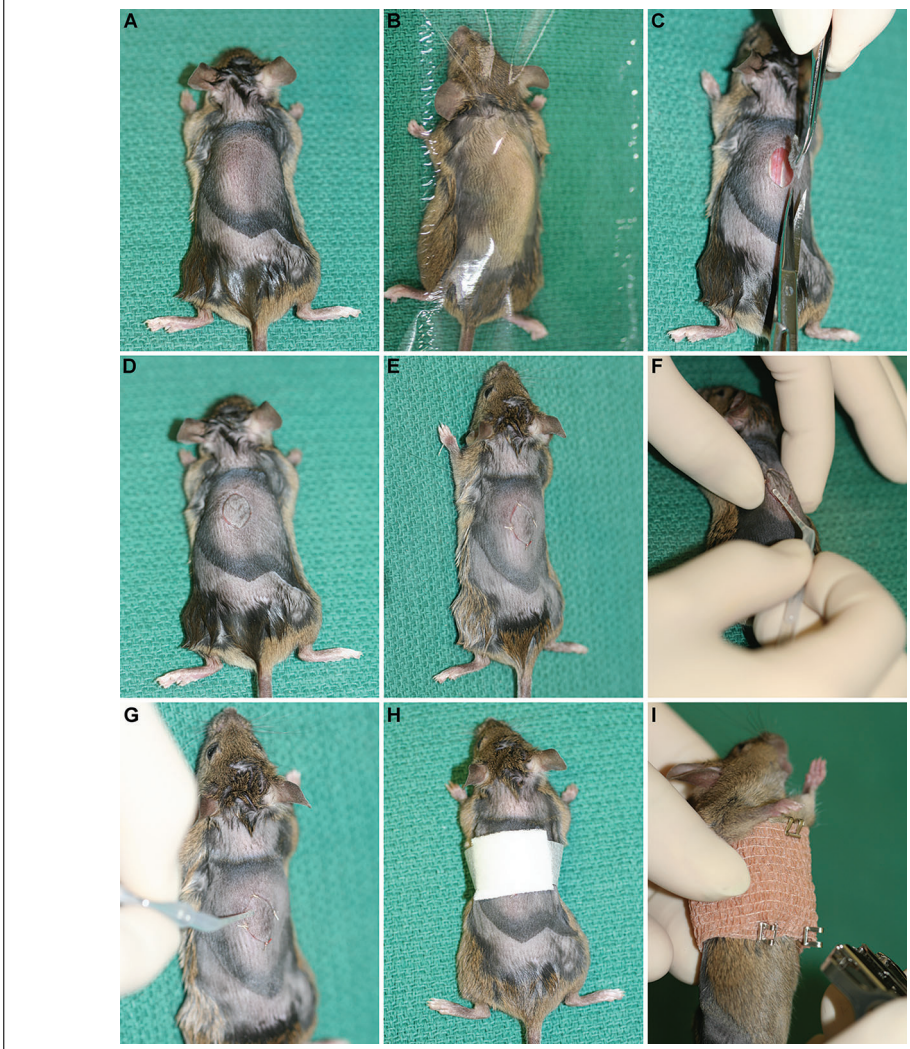

Figure 2 Surgical procedure. (**A**) The anesthetized mouse is laid in ventral recumbency. The dorsal skin is shaved and surgically disinfected. The dark areas of skin have hair follicles in anagen whereas the light areas are in telogen, both of which are normal. (**B**) The surgical site is draped, with the drape remaining on throughout the procedure (drape removed for demonstration only in this image series). (**C**) The skin is raised (tented) using forceps and excised with curved iris scissors. (**D**) The donor skin is rotated 180° and placed in the wound site of the recipient mouse (for the controls this will allow observation of hair growth opposite that of normal). (**E**–**G**) The graft is sutured at the four corners (**E**) and then the edges are glued (**F and G**). (**H**) A nonstick dressing pad is applied over the site and taped in place. (**I**) Coban is wrapped completely around the thorax and fixed in place with wound clips. Illustrations used with approval (Silva & Sundberg, 2013).

Mouse recovery

24. After surgery, place mice in ventral recumbency on a clean paper towel within the cage.

25. Administer sterile saline (1-2 ml per 10 g body weight) subcutaneously to correct for any fluid deficits.

26. Place a 40-W light bulb over one end of the cage to provide warmth until the mice have made a complete recovery from anesthesia.

    To prevent overheating, mice should never be placed directly under the light. Placing and monitoring a thermometer within the cage will ensure mice do not overheat.

27. After mice have completely recovered from anesthesia, transfer them to an isolator cage.

28. Provide sulfamethoxazole (200 mg sulfamethoxazole plus 40 trimethoprim in 225 ml water) in the drinking water for 1 week after surgery to minimize the risk of infection.

29. Observe mice daily for the first week after surgery to make sure that they have not removed the bandage or destroyed the graft site and are eating, drinking, and behaving normally.

    The dressing pad and self-adhering bandage can be reapplied if the bandage was removed.

30. Observe each mouse for signs of pain or distress and treat accordingly as needed. Mice in distress or pain can be given carprofen every 24 hr for 2-3 days.

    Aggressive behavior when handled, decreased food and water intake, decreased activity, hiding, and burrowing are all indicators of distress or pain. To encourage the mice to eat, food pellets or moistened crushed food pellets can be placed on the bottom of the cage.

31. Remove bandages, sutures, and wound clips 7-9 days after surgery. The graft recipient mice should be separated into individual cages for 7-10 days after surgery so that the mice do not remove the unhealed grafts from each other.

32. Monitor the graft sites for hair growth or loss during the subsequent 20-week period.

    Typically, we observe that 90% or more mice develop AA over this period.

ADOPTIVE TRANSFER OF CULTURED LYMPHOID CELLS PROVIDES A NONSURGICAL METHOD TO INDUCE ALOPECIA AREATA IN C3H/HEJ MICE

BASIC PROTOCOL 2

Although the skin-grafting technique is an effective and reliable method of transferring AA to recipient mice, and produces large numbers of mice for research, an alternative cell injection method of AA transfer was subsequently developed. In part, this development was driven by more restrictive animal ethics limitations in several countries (such as Canada and the UK) as compared to the United States. Consequently, alternative approaches were developed based on observations that fresh cells from skin-draining lymph nodes, and to a lesser extent spleens, of AA-affected mice could be injected into normal-haired recipients and transfer the AA phenotype (Carroll et al., 2002; Wang et al., 2015). A cell culture methodology was developed to increase the number of lymph node cells obtained from a single spontaneous AA donor mouse, such that many young normal-haired recipient mice could be injected with cells to transfer the AA phenotype.

Materials

Sterile phosphate-buffered saline (PBS), pH 7.4 (Mediatech Inc., Manassas, VA, cat. no. 46-013-CM)
0.5 M ethylenediaminetetraacetic acid (EDTA), pH 8.0 (Ambion, Burlington, ON, Canada, cat. no. AM9260G)
Sterile Dulbecco's phosphate-buffered saline (DPBS), pH 7.0 (Sigma, Oakville, ON, Canada, cat. no. D1408)
Advanced RPMI 1640 (Gibco, Burlington, ON, Canada, cat. no. 12633-012)
Fetal bovine serum (FBS; Gibco, cat. no. 16000044)
GlutaMAX (Gibco, cat. no. 35050-061)
Penicillin-streptomycin (Gibco, cat. no. 15140-148)
Human Recombinant IL2 (Roche Life Science, Laval, QC, Canada, cat. no. 11011456001)

Mouse Recombinant IL7 (R&D Systems, Minneapolis, MN, cat. no. 407-ML-005)
Mouse Recombinant IL15 (R&D Systems, cat. no. 447-ML-010)
70% (v/v) ethanol
Female C3H/HeJ donor mice with extensive AA
Trypan blue (Invitrogen, Burlington, ON, Canada, cat. no. 15250-061)
Cell recipient 8-week-old female mice: e.g., Jackson Laboratory strain no. 000659 (The Jackson Laboratory, Bar Harbor, ME; *http://jaxmice.jax.org/*)
Anesthesia: Isoflurane (Covetrus, Dublin, OH)
Dynabeads Mouse T-Activator CD3/CD28 (Gibco, cat. no. 114.52D)

EasySep™ Magnet (StemCell Technologies, Vancouver, BC, Canada, cat. no. 18000)
Sterile round-bottom Falcon tubes, 12 × 75 mm (VWR, Mississauga, ON, Canada, cat. no. 734-0445)
Euthanasia method (CO_2 or other method with appropriate Institutional Animal Care and Use Committee (IACUC) or appropriate institutional approval)
Electric clippers (Oster Finisher Trimmer, Oster Professional Products, Newell Brands, Atlanta, GA USA, cat. no. 76059-030)
Surgical scissors and forceps (Roboz Surgical Instrument, Gaithersburg, MD), sterile
Petri dishes, 35 × 10 mm (Fisher Scientific, cat. no. 08-757-100A)
Type II biosafety cabinet
70-µm-pore-size cell strainers (Fisher Scientific, Ottawa, ON, Canada, cat. no. 08-771-2)
1-cc insulin syringes (BD, Mississauga, ON, Canada, cat. no. 329420)
15- and 50-ml centrifuge tubes (Fisher Scientific)
Improved Neubauer Hemocytometer (Fisher Scientific)
Inverted microscope (Nikon Instruments, Melville, NY)
Centrifuge capable of up to 600 × g (Thermofisher, San Diego, CA)
Non-treated 24-well tissue culture plates (BD Biosciences, Mississauga, ON, Canada, cat. no. 351147)
37°C, 5% CO_2 incubator (NuAire, Plymouth, MN)
25- and 75-ml (T25 and T75) suspension cell tissue culture flasks, vented (Sarstedt, Montreal, QC, Canada, cat. nos. 83-1810-502 and 83-1813-502)
Heat pad or heat lamp (use method approved by institutional animal welfare committee)

Reagent preparation

Make all reagents fresh immediately prior to use, if not aliquoted and stored as described.

1. Prepare Dynabead wash buffer by mixing 0.1% FBS and 2 mM EDTA into sterile phosphate-buffered saline (PBS).
2. Prepare 1× PBS by diluting with sterile distilled water.
3. Prepare 1× DPBS by diluting with sterile distilled water.
4. Prepare complete AR10 medium consisting advanced RPMI 1640, 10% FBS, 2 mM GlutaMAX, and 100 U/ml penicillin-streptomycin.
5. Prepare mouse recombinant IL7 by reconstituting as 25 ng/µl in sterile PBS with 0.1% FBS. Aliquot and store at −20°C.
6. Prepare mouse recombinant IL15 by reconstituting as 50 ng/µl in sterile PBS with 0.1% FBS. Aliquot and store at −20°C.
7. Prepare complete AR10 medium supplemented with cytokines, consisting of AR10 (step 7) supplemented with 30 U/ml recombinant human IL2, 25 ng/ml recombinant mouse IL7, and 50 ng/ml recombinant mouse IL15.

8. Prepare Dynabead mixture by following the manufacturer's instructions: Resuspend the Dynabeads by vortexing for 30 s. Pipet the Dynabeads into a sterile round-bottom Falcon centrifuge tube and add 1 ml Dynabead wash buffer (step 1). Then pipet gently up and down ~30 times, being careful to avoid generating air bubbles.

   A 25-µl volume of this Dynabead preparation will be needed for each 1 million lymph node cells isolated from the AA-affected donor mouse.

9. Place the centrifuge tube into an EasySep magnet for 1 min, and remove the wash buffer while keeping the tube inside the magnet. The Dynabeads will "adhere" to the wall of the tube.

10. Carefully remove the tube from the magnet and add AR10 supplemented with cytokines at 500 µl/25 µl of Dynabeads. Confirm that all of the Dynabeads on the tube wall are resuspended into the medium, ready for use.

Isolation of skin-draining lymph nodes

11. Euthanize donor mice with extensive AA according to the recommendations of the American Veterinary Medical Association (Leary et al., 2020), or national equivalent, with approval by the appropriate regulatory committee where the work will be done.

    Representative areas with alopecia should be biopsied to confirm the diagnosis as other diseases can cause alopecia in mice (Sundberg et al., 2022).

12. Clean the mouse skin with 70% ethanol.

13. Place the mouse in dorsal recumbency on a flat surface, such as a corkboard covered with a paper towel, and pin in place using tacks.

14. Proceed with ventral incisions from the midline laterally over front and rear legs, and then a midline incision the length of the torso, using sterile scissors.

15. Reflect the skin to expose the inguinal, axillary, and cervical skin-draining lymph nodes.

16. Trim away fat and connective tissue overlaying the lymph nodes using fresh sterile instruments.

17. Remove and place the skin-draining lymph nodes in complete AR10 medium on ice. To prevent tissue drying, process lymph nodes (subsequent steps) as quickly as possible.

    This procedure should take 30 min per mouse at most.

Separation of lymph node cells (LNCs) into a single-cell suspension

This work should be done in a type II biosafety cabinet using aseptic techniques.

18. Fill a small petri dish with 1 ml of 1× DPBS.

19. Transfer the lymph nodes from the complete AR10 medium into DPBS and wash three times by briefly rinsing with fresh DPBS.

20. Add 1 ml fresh complete AR10 medium into a clean, sterile petri dish. Place a 70-µm cell strainer in the middle of the petri dish.

21. Transfer the lymph nodes into the middle of the cell strainer with a pair of forceps.

22. Gently mash the lymph nodes against the cell strainer using the plunger end of a 10-cc syringe for ~5 min.

The culture medium will become opaque as the lymph nodes are broken apart, resulting in a small residue consisting of pale-colored connective tissue in the strainer. It is important to change the angle and direction of grinding the lymph nodes to ensure complete breakdown of the tissues and to recover the maximum number of LNCs.

23. Rinse the bottom of the cell strainer with 1 ml fresh complete AR10 medium to remove any extra LNCs into the petri dish.

    This should result in ~2 ml of LNC suspension.

24. Remove the cell strainer. Transfer the LNC suspension into a clean, sterile 15-ml centrifuge tube.

25. Wash the petri dish with another 1 ml fresh complete AR10 medium and add this to the centrifuge tube.

26. Fill the centrifuge tube to 10 ml total by adding another 7 ml fresh complete AR10 medium and pipet thoroughly, but gently, to wash the cell suspension.

27. Remove 10 µl of the cell suspension. Count the number of cells present using a hemocytometer and trypan blue (or equivalent cell counting system) and determine how many cells will be used for expansion.

    A minimum of 10 million cultured cells are required for injection into a single recipient.

28. Prepare fresh complete AR10 medium with cytokines (see step 7), making enough to resuspend the cell pellet to a concentration of 2×10^6 cells per ml.

29. Centrifuge the cell suspension for 5 min at $350 \times g$, room temperature.

30. Decant to remove the supernatant while leaving the cell pellet.

31. Resuspend the cell pellet (2×10^6 cells per ml) with complete AR10 medium supplemented with cytokines (step 28).

32. Pipet 1 ml of the cell suspension into a non-tissue-culture-treated 24-well plate to each of the desired number of wells. When filled, each well should contain 2×10^6 cells.

33. Set aside the 24-well plates in a 37°C, 5% CO_2 incubator until ready for the next step.

34. Prepare the Dynabead mixture (step 1). Remove the plates from the incubator and gently resuspend the cells in each well with 500 µl of the Dynabead mixture. Each well should now contain 2×10^6 cells in 1.5 ml of AR10 medium supplemented with cytokines and Dynabeads.

    Keeping the LNCs at a high density throughout the culturing process results in better cell-to-cell contact and interaction between the cells and the antibody-coated magnetic beads. Maintaining a high cell density is particularly important in determining the success of cell proliferation!

35. Place the plate back into the 37°C, 5% CO_2 incubator. Cell cultures can be monitored with an inverted microscope at time points described below.

    When removing and replacing the plates in the incubator, be careful not to shake or otherwise agitate the cells. Doing so seems to reduce the rate of cell proliferation.

    This cell culture preparation procedure should take ~1 hr to perform.

Expansion and activation of LNCs

36. Evaluate the culture daily to make sure there is no contamination, but avoid disturbing the LNC suspension. After 24 hr, clumping of LNCs with the Dynabeads may be observed.

Do not attempt to break up the clumps; these will disappear after 48 hr. The LNCs should expand to cover the bottom of each well, and after 48-72 hr the culture medium should have a light-yellow coloration.

37. Split each well into two by gently resuspending the culture, pipetting 750 µl to an adjacent well, and then adding 750 µl fresh complete AR10 medium supplemented with cytokines. Each well should now contain 1.5 ml. Place plates back in the incubator.

38. After a further 24-hr incubation, combine two wells into one T25 flask. To this add 3 ml of complete AR10 medium supplemented with cytokines.

39. After an additional 24-hr incubation period, combine the cells from two T25 flasks into one T75 flask and add 8 ml of complete AR10 medium supplemented with cytokines.

40. Culture the T75 flasks for an additional 24 hr.

    The cell numbers should expand 8-10× over the total 6-7 day period.

41. Keep the LNCs at very high density, ∼1.5-2 million cells/ml, throughout the cell culture process. This protocol provides general guidelines only.

    Change the time durations accordingly; the priority is maintaining high cell density, and time duration is secondary.

    This procedure will typically take 6 days, but this can vary depending on the rate of cell proliferation in the first 48 hr.

Preparation of expanded LNCs for injection

42. Combine the LNC suspensions in T75 flasks into one or more 50-ml centrifuge tubes.

    Although cells from more than one flask may be pooled together for processing, in our experience, cells from different donor mice should not be mixed together for injection into recipients. Even though C3H/HeJ mice are fully inbred, combining cells from different donors reduces success in transferring AA.

43. Place a clean round-bottom Falcon centrifuge tube into an EasySep magnet or similar device.

44. Transfer all of the LNC suspension into the centrifuge tube to a level at the same height as the magnet and let it rest there for at least 1 min.

45. Gently pipet the LNC suspension from the centrifuge tube into a clean 50-ml centrifuge tube.

46. Repeat until all Dynabeads have been removed from all LNC suspensions. Keep the centrifuge tube inside the magnet while transferring the LNC suspension to a new 50-ml tube.

    Do not scratch the wall of the tube while pipetting, as the Dynabeads will come off and go back into the LNC suspension.

47. Remove 10 µl and count cells with a hemocytometer. If the cell recovery rate is low, even though expansion was noted, wash the plate or flask again with fresh complete AR10 medium to remove any cells that may have adhered to the bottoms of plates or flasks.

48. Centrifuge the LNC suspension for 5 min at 350 × *g*, room temperature. Red precipitates may be observed within the cell pellets after centrifugation if the Dynabeads were not completely removed.

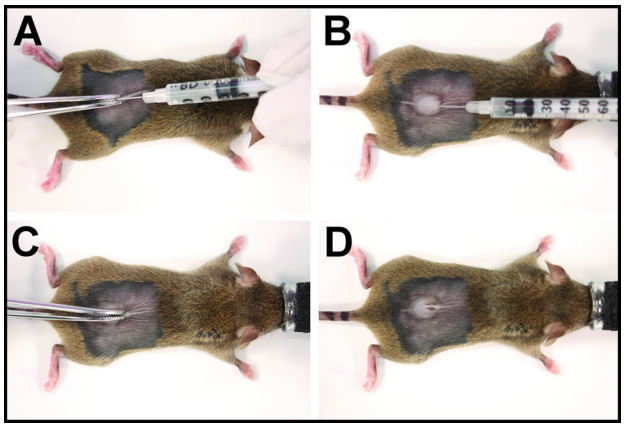

Figure 3 Lymph node cell injection method. (**A**) Anesthetized mice have their dorsal skin surgically prepared (shaved and disinfected). The skin is gently tented using forceps and the needle of the syringe is inserted into the skin parallel to the pinched skin. This reduces the chance of cells entering the subcutaneous tissues: some cells are successfully injected into the dermis, although most end up in the fat layer (Liang et al., 2011; Wang et al., 2015). (**B**) 10^7 lymph node cells in 100 µl of phosphate-buffered saline are slowly injected into the tented skin to form a bulla. (**C**) Blunt forceps are used to clamp the needle wound to briefly close the wound. (**D**) The bulla will slowly dissipate into the surrounding skin. Illustrations used with approval (Wang & McElwee, 2020).

49. Decant the supernatant from the LNC pellet and resuspend with sterile PBS to a concentration of 1×10^7 cells per 100 µl.

50. Aspirate the resuspended LNC suspension into an insulin syringe or similar. Keep the syringe on ice until used.

    Insulin syringes and needles are sealed as one unit, which eliminates the problem seen with small volumes that they can be difficult to eject from conventional syringes and needles.

51. Inject the LNCs as soon as possible after filling the syringes. If this cannot be done, then resuspend the cell pellets with 1-2 ml of PBS in the round-bottom centrifuge tubes and place them back into the magnet for another 2 min, repeating the wash step.

    This cell preparation procedure will take ~1.5 hr to complete.

Intradermal injection of LNCs into recipient C3H/HeJ mice (adoptive transfer)

52. Anaesthetize the recipient mice (make sure these are of the same strain and sex to avoid any major or minor histoincompatibilities), ideally 10 weeks old, using isoflurane or other anesthesia, following protocols approved by the local institutional review board.

    Mice <8 weeks old are relatively resistant to AA induction using cell injections.

53. Shave a small area on the back of the mice to expose an area of skin for injection. Disinfect the area with 70% ethanol immediately before injection.

54. Gently pinch the skin with a pair of blunt forceps, insert the syringe needle almost parallel to the plane of the tented skin into the dermis layer (Fig. 3), and inject 100 µl of the LNC suspension slowly into the skin until a small bulge appears at the injection site.

    LNCs injected deep into the subcutaneous layer may result in lower AA induction success rates.

55. Retract the syringe and use the forceps to hold the skin for a few seconds to allow the injected LNCs to slightly dissipate and close the point of injection.

56. Place the injected mice back into a cage, taking care not to press on the injection site.

57. Place a heat lamp above the mouse cage until they are fully recovered as discussed in Basic Protocol 1. Mice will typically recover from isoflurane anesthesia within 10 min.

This procedure, from shaving to cage return, will take ~5-10 min per mouse to complete.

COMMENTARY

Background Information

Regardless of the method used, histopathological evaluation of biopsies taken from donors and recipients is important to confirm that the hair loss observed is due to AA and not unrelated diseases. Histologic parameters are described in detail elsewhere (Pratt et al., 2017; Sundberg, Cordy, & King, 1994; Sundberg et al., 2022).

With either method, AA develops initially as patchy alopecia and progresses over 20 weeks to near-total alopecia (Fig. 4). Responses to manipulation can be semi-quantified by a variety of methods, one of which is illustrated in Fig. 5 (Sundberg et al., 2019).

The C3H/HeJ mouse AA model, induced by either full-thickness skin grafts or cell transfer, has yielded a highly reproducible tool to work out the pathogenesis of this complicated and relatively common human disease. Although creating mice is more difficult that simply ordering mice from a vendor, it has been critical to advancing our understanding of the pathogenesis of AA (Carroll et al., 2002; Pratt et al., 2017) and has enabled successful preclinical trials that led to the first approved new therapy for AA (Dai et al., 2021; King et al., 2022; Wang et al., 2018; Xing et al., 2014).

Critical Parameters

Basic Protocol 1

Several elements of the protocol are particularly important to ensure robust and reproducible results in transferring AA to normal-haired recipients using skin grafts from spontaneously AA-affected mice. Selecting donor mice with well-characterized spontaneous AA provides a consistent skin graft disease model. Although there seems to be little difference in response rates whether using donor skin grafts from a patchy AA-affected mouse or a mouse with near-universal AA, mice with slight, diffuse hair loss are best avoided as donors, as they tend to give poor results in transferring AA to graft recipients.

Using healthy, age-matched littermates as recipients minimizes variability in immune response. Although there is no clear evidence that old normal-haired graft recipient mice are less likely to develop AA, their limited remaining lifespan makes them difficult to use in longer-duration experiments. In early investigations, it was found that recipient mice grafted at 6 weeks of age and younger were more resistant to the onset of AA (unpublished observations), and they are less suitable as graft recipients. Mice

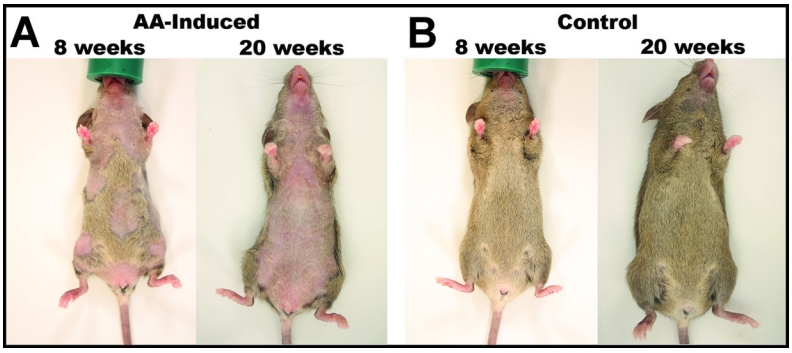

Figure 4 Onset of alopecia areata. (**A**) Regardless of induction method, mice develop patchy alopecia initially on the ventral skin in the inguinal and then axillary regions that progresses to the dorsal skin, eventually resulting in near-total loss of hair. (**B**) Induction with normal donor skin in the graft model or normal lymph node cells in the cell transfer model results in mice remaining fully haired. Illustrations used with approval (Wang & McElwee, 2020).

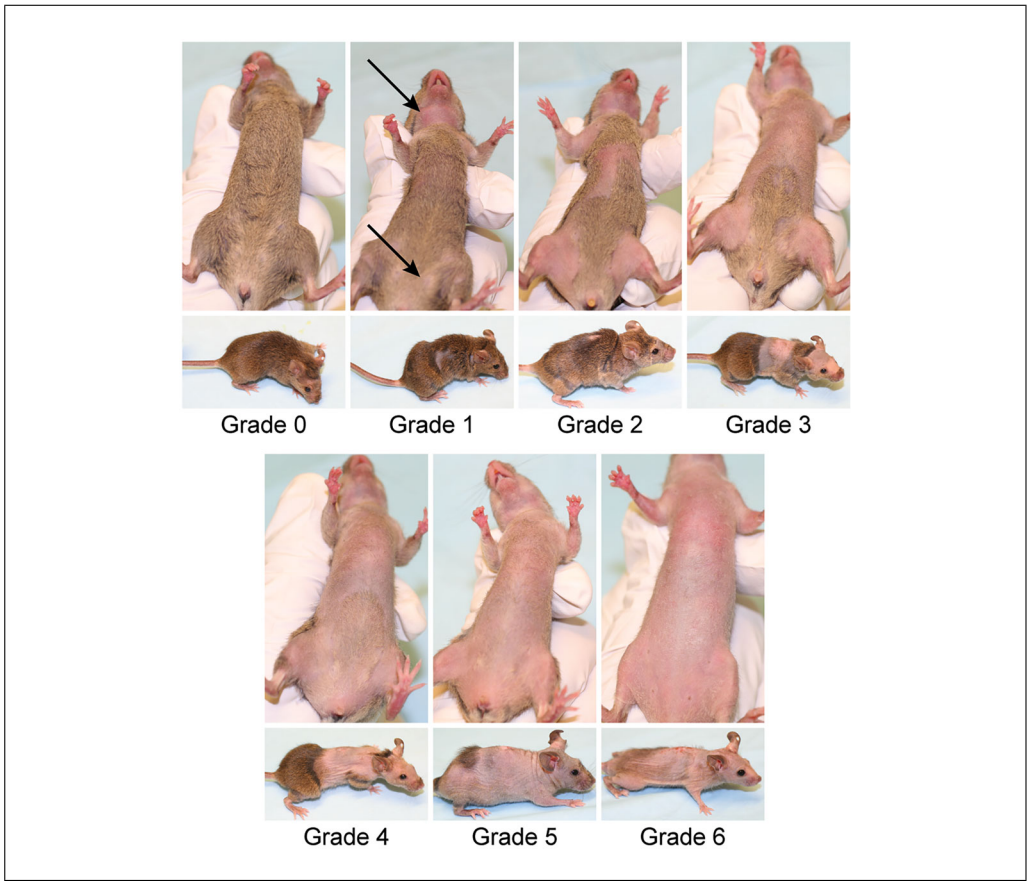

Figure 5 Grading disease progression of alopecia areata in C3H/HeJ mice. Regardless of induction method, disease will progress over 20 weeks after induction. Results of treatment, whether with drugs, antibodies, or other approaches to modulate the disease, can be observed by grading lesions on a weekly basis using a subjective scale. Image analysis can yield more analytical data. Illustrations used with approval (Sundberg et al., 2019).

aged 10-12 weeks at the time of skin grafting are therefore preferred for optimal AA induction results and long-term, repeated observation studies. Mice should be kept on a low-fat diet, as high-fat/oil diets have been found to reduce the success rates of skin graft AA induction (McElwee, Niiyama, Freyschmidt-Paul, et al., 2003).

More general surgical parameters are relevant in facilitating successful AA initiation. Precise and sterile surgical techniques are necessary to minimize infection and trauma, which can affect graft survival and AA transfer success. Variability in surgical technique, such as inconsistent graft placement or handling, can lead to different outcomes in graft acceptance. Maintaining consistent environmental conditions for grafted mice, including temperature, humidity, and light cycles, may be relevant as previous research has shown that stress can influence immune responses and could potentially interfere with uniformity of results. Differences in post-operative care, such as analgesic administration, wound care, and monitoring, can also impact the general health and recovery of the grafted mice.

Additionally, the timing and frequency of observations and assessments must be consistent to accurately capture the onset and progression of AA. Finally, inconsistent handling of the mice can induce stress, which may alter immune responses and affect the experiment's outcome. By focusing on these key elements, the reliability and interpretability of findings in AA studies can be enhanced.

Basic Protocol 2

This second protocol was developed as a way to produce AA on a large scale in the C3H/HeJ mouse model while avoiding the use of skin-grafting surgical techniques. Surgery requires training, is a relatively labor-intensive process, and requires close monitoring of grafted mice to maintain good standards of post-surgical care. It also involves anesthetics, analgesics, equipment, and a surgical theater that may not be readily available. Consequently, in some countries, it can be difficult

to obtain ethical approval for skin-grafting methodology. Cell culture and injection to induce AA in normal-haired recipients may be a simpler approach that draws on the prior laboratory experience of those involved. Which protocol is most appropriate to use very much depends on the knowledge and facilities available to the researcher and must be determined based on individual circumstances. Note that the overall costs of Basic Protocols 1 and 2 are similar, in the authors' experience.

For successful completion of Basic Protocol 2, the quality of LNCs derived from the donor as the initial cells for amplification are arguably the most important factor. Ideally, LNCs are harvested from spontaneous-AA-affected donors with 50-80% hair loss to ensure that they carry the disease phenotype effectively. Allowing the mice to progress to more extensive AA also allows time for LNCs to accumulate in draining lymph nodes, such that a greater number can be obtained for seeding cultures. Mice with chronic near-universal AA, and/or AA-affected mice older than 12 months of age, can also be used as donors. However, aged mice tend to reach a stable disease state in which somewhat fewer LNCs can be retrieved from draining lymph nodes. The composition of the cell population retrieved might also be different, though this has not been properly investigated. The number of fresh LNCs harvested from each spontaneous AA donor mouse is variable. It should be possible to obtain ~50-90 $\times$ 10^6 cells from each donor.

Cell culture conditions are particularly important for successful production of this AA model. In the development of the protocol, expansion and activation of LNCs with IL2, IL7, and IL15, along with anti-CD3/anti-CD28 magnetic beads, were each found to be required to allow significant expansion of cell numbers while maintaining the cell's capacity to induce AA. High cell density must be maintained at all times to achieve optimal cell expansion and activation. Using larger culture plates, accidentally knocking the culture plates when assessing them under a microscope, or intentionally breaking up the cell-bead clusters during their proliferation phase all significantly reduce the final cell count achieved. Scientists new to the culture protocol often query the apparent lack of nutrient availability in maintaining such high cell densities. However, any attempts to add larger volumes of medium, consequently reducing cell density, reduces the final cell count. Maximal cell-bead physical contact is the paramount requirement at all times throughout the cell culture process.

It is important to feed recipient mice a low-fat diet. High-fat/oil diets have been found to reduce the success rates of AA by cell injection (unpublished observations), similar to observations with skin-graft-induced AA (McElwee, Niiyama, Freyschmidt-Paul, et al., 2003). If the injected mice do not develop any hair loss within 20 weeks, there are several possibilities to explain the results. If the female C3H/HeJ were used too young, this is often a problem. Minor histocompatibility issues may play a role, especially if using different-sex mice (H-Y antigens). Do not inject male-derived LNCs into female recipients; the injected cells may be rejected (female-derived LNCs injected into male recipients should be accepted). Sometimes, when using C3H/HeJ or C3 congenic strains carrying specific spontaneous or induced mutations, the phenotype might affect the immune response.

Troubleshooting

There are various problems that can arise with the methodology for skin grafting to induce AA. Some of the more common issues that may be encountered are outlined in Table 1.

There are various problems that can arise with the methodology for LNC cell injection to induce AA. Some of the more common issues that may be encountered are outlined in Table 2.

Understanding Results

Basic Protocol 1

Surgical failures can be due to loss of the skin grafts when mice remove bandages and destroy the grafted skin. Similarly, infection of the wound site can result in skin graft rejection. Consequently, good aseptic surgical procedure can have a significant impact on any study utilizing the AA grafting technique. Possibly, speed of the surgical procedure, specifically the time between taking the donor grafts and grafting them to recipients, may also affect success rates (unpublished observations).

Occasionally, C3H/HeJ mice may accept their grafts but fail to develop AA. It should not be assumed that these mice have no immune response or can be used as control mice in subsequent experiments. Previous studies have shown that mice that accept AA skin grafts, but fail to develop AA, exhibit an active immune resistance to AA development, with local skin infiltration of antigen-presenting cell populations and high levels of IL10

Table 1 Troubleshooting Guide for Basic Protocol 1

Problem	Possible cause	Solution
No spontaneous AA in candidate donor mice	Candidate donor mice that were too young	Age donor mice up to 14 months and wait for onset of spontaneous AA, with first onset most commonly at 6-9 months of age
Low rate of spontaneous AA in candidate donor mice	Candidate donor mice that were given high-fat diet	Ensure mice are given a diet that is ≤4% fat
	Mice that were not retired breeders	Use retired female breeder mice as the candidate donors that will develop AA; breeding increases penetrance of the clinical AA phenotype
Graft wound dehiscence	Mice attacking skin grafts	House mice one per cage for up to 7 days post grafting to avoid mice attacking grafts
Graft rejection	Wound glue preventing skin healing	Avoid using wound glue around the entire graft as this can block contact between graft and recipient skin
	Infection	Ensure that recipient mice are given antibiotics to reduce chances of wound infection
	Infection due to mice removing bandages prematurely	Monitor mice closely after grafting, and reapply bandages should a mouse remove the original bandage
	Male donor skin grafted to female recipients	The presence of Y-chromosome gene expression in male skin can elicit graft rejection by female mice—only graft female recipients with female donor skin
	Graft not histocompatible	Some genetically induced mutations in C3H/HeJ mice may prove not to be histocompatible with wild-type mice
Failure to induce AA in graft recipients	Skin graft donor did not have AA	Ensure that alopecia observed in candidate graft donors is indeed AA, as barbering can sometimes produce patches of hair loss that can superficially look like AA
Low rate of AA in graft recipients	Recipient mice were given high-fat diet	Ensure graft recipient mice are given a diet that is ≤4% fat
	Recipient mice were too young	Mice younger than 6 weeks of age at the time of grafting are much less likely to develop AA—ensure that graft recipient mice are aged ≥8 weeks and ideally 10-12 weeks

cytokine production (McElwee et al., 2002). This observation was subsequently attributed in part to interference in the AA transfer process due to a diet high in soy oil (McElwee, Niiyama, Freyschmidt-Paul, et al., 2003), though other, unknown inputs are likely involved.

Failure of AA development in graft recipients with successful alopecia skin grafts onto genetically engineered mice, especially if all mice in a study do not lose hair, may indicate the identification of a disease mechanism, and not necessarily a surgical failure (Carroll et al., 2002). In addition, the time duration until clinical onset of AA in genetically engineered mice may be significantly different from that in wild-type C3H/HeJ mice, depending on the modification.

Control mice may develop AA spontaneously, as this is a natural strain-specific disease (background rate of ∼15%-25% in mice 12 months of age and older). However, spontaneous AA rarely appears at 6 months of age or earlier (Sundberg, Cordy, & King, 1994). Control mice that develop AA should be noted in data, but should not be used as controls in research analysis (histology, flow cytometry, etc.); however, this situation should be rare because the recipient mice should be relatively young.

Success rates in transferring AA with the skin graft procedure vary with geographic location. At The Jackson Laboratory, Bar Harbor, ME, the success rate can be nearly 100% of recipients developing AA. Surgical skill can be a major factor. Success rates in other

Table 2 Troubleshooting Guide for Basic Protocol 2

Problem	Possible cause	Solution
Low rate of spontaneous AA in candidate donor mice	Candidate donor mice that were given high-fat diet	Ensure donor mice are given a diet that is ≤4% fat
	Mice that were not retired breeders	Use retired female breeder mice as the candidate donors that will develop AA; breeding increases penetrance of the clinical AA phenotype
Small draining lymph nodes dissected or low numbers of skin-draining LNCs retrieved	Individual donor that did not have AA	Ensure that alopecia observed in candidate donors is indeed AA, as barbering can sometimes produce patches of hair loss that can superficially look like AA
Low numbers of skin-draining LNCs retrieved	Failure to remove fat from around draining lymph nodes before isolating LNCs	Dissect all fat from around the lymph nodes to enable complete breakdown of the tissues and to recover maximum number of LNCs
	Slow processing of donor lymph nodes to isolate LNCs	Prolonged processing of lymph nodes can reduce the number of LNCs retrieved; speed of processing is relevant
	Lack of "vigor" in processing of donor lymph nodes to isolate LNCs	Change the angle and direction while grinding the lymph nodes to ensure complete breakdown of the tissues and recover maximum number of LNCs
Poor cell proliferation in culture	Low cell density during culture	Maintaining a high cell density is particularly important in determining success of the cell proliferation phase—the goal being to keep the LNCs at ∼1.5-2 million cells/ml at all times.
	Cells from more than one donor mixed together	In the authors' experience, mixing cells from more than one donor significantly reduces cell proliferation (for unknown reasons)
Failure to induce AA in LNC recipients	Male donor cells injected into female recipients	Y-chromosome gene expression in male LNC cells can elicit cell rejection by female mice—only inject female recipients with female donor LNCs
Low rate of AA in LNC recipients	Recipient mice given high-fat diet	Ensure recipient mice are given a diet that is ≤4% fat
	Recipient mice that were too young	Mice younger than 6 weeks of age at the time of cell injection are much less likely to develop AA—ensure that recipient mice are aged ≥8 weeks
	Recipient mice that were injected subcutaneously	Ensure mice are injected with LNCs intradermally—subcutaneous injection significantly reduces the rate of AA induction in recipients

locations, even when using mice supplied by The Jackson Laboratory, may be lower, based on observations in Canada, Germany, and the UK (McElwee, unpublished observations). Diet has been shown to be very important in determining the development of AA in graft-recipient mice. Mice fed a 4% soy oil/fat diet were more likely to develop AA than mice on a 6% soy oil/fat diet from the same supplier. A specially made 20% soy oil/fat diet fully inhibited AA development in skin-grafted mice in published research (McElwee, Niiyama, Freyschmidt-Paul, et al., 2003). It is likely that stress levels in the animal facility also impact AA transfer success (Zhang et al., 2009). Other, as yet unresearched inputs are also likely to contribute.

Basic Protocol 2

By using the optimal parameters described above, including careful selection of AA LNC donors, maximal retrieval of cells from skin-draining lymph nodes, and culture of cells closely following the described protocol, a single spontaneous AA donor can yield 50-90 $\times$ 10^7 cells, which would enable intradermal injection of 50-90 recipient mice. Among injected mice, maintained under optimal

conditions, up to 90% (45-80 mice) should develop overt AA. These mice can then be subdivided into cohorts for research investigation. Basic Protocol 2, and variations thereof, have been used to produce AA mice for several studies (Hashimoto, Yamada, Fujikawa, et al., 2021; Hashimoto et al., 2022; Ito et al., 2020; Lee et al., 2022; Wang et al., 2015). Although the AA induced by skin grafting (Basic Protocol 1) has not been directly compared to the AA induced by adoptive cell transfer (Basic Protocol 2), the published studies suggest that the AA phenotypes are comparable.

If needed, lymph node cells from normal-haired mice can also be processed in the same way to produce a negative comparative control cohort for baseline comparison. However, lymph nodes in healthy mice are much smaller, such that only $\sim$2-4 $\times$ 10^6 cells can be obtained per donor. Consequently, far fewer control mice can be produced from a single donor using the same culture protocol. In the authors' experience, mice injected with cells derived from healthy, non-AA-affected mice do not develop AA, though isolated cases of spontaneous AA development should be anticipated (Wang et al., 2015).

Hashimoto and colleagues have examined the composition of the lymph node derived cells before and after using the cell culture protocol component of Basic Protocol 2 described above (Hashimoto, Yamada, Sekiguchi, et al., 2021). The percentage of effector memory $CD8^+$ T cells doubles, reaching $>$50% of $CD3^+$ T cells after 7 days in culture. Correspondingly, the number of $CD4^+$ cells, initially $\sim$50% of freshly isolated lymph nodes cells, drops to $<$20% of cells after culture. The number of $NKG2D^+$ Tc1 cells was 0.9%, whereas $CD11c^+$ dendritic cells comprised 2.9% of the total cell population at the end of culture (Hashimoto, Yamada, Sekiguchi, et al., 2021). Similar data have been independently obtained by others (Lee et al., 2022). The data are consistent with CD8 and CD4 lymphocytes being the main AA transfer agents in this model.

The published data indicate that mouse AA is driven by immune cells and that skin-draining lymph nodes of spontaneous-AA-affected donors provide an enriched source of pathogenic cells. Potentially these cells could be separated into specific populations for study, and/or recombined in specific combinations for injection into recipients for functional analysis. McElwee and Co. showed that $CD8^+$, and separately $CD4^+/CD25^-$, cells can induce AA (McElwee, Freyschmidt-Paul, Hoffmann, et al., 2005). Xing et al. demonstrated $NKG2D^+/CD8^+$ cells were the primary drivers of hair loss in AA mice (Xing et al., 2014). Ito and colleagues have used a similar approach to reveal a significant role for plasmacytoid dendritic cells in AA development (Ito et al., 2020). Interestingly, the ability to induce AA in apparently normal-haired recipients by cell transfer suggests that the apparent immune privilege of hair follicles (Bertolini et al., 2020) provides little or no resistance against activated pathogenic AA immune cells.

Time Considerations

Basic Protocol 1

The skin graft wounds usually heal uneventfully within 2 weeks. Mice regrow hair within the shaved surgical sites for both AA donor and control/sham donor skin. For mice that receive AA donor skin, hair loss usually is initially observed adjacent to the skin graft site as well as in the axillary and inguinal regions of the ventral skin as early as 5 to 6 weeks after surgery, usually by 8 to 10 weeks, and occasionally as late as 11 to 12 weeks. Alopecia of the ventral skin expands centrifugally, as patchy alopecia develops on the dorsal skin. Many, but not all, mice are extensively alopecic (near alopecia universalis), with just isolated stubble remaining, by 20 weeks after surgery.

Basic Protocol 2

The cell expansion rate in culture is variable between donors; usually changes in cell morphology can be observed at 48 hr at a minimum. By 72 hr, the cells should cover $>$90% of the bottom of the 24-well plate. Time duration of culture steps should be adjusted with maintenance of a high cell density and cell-cell and cell-bead contact being the priority at all times. However, beyond 6 days of culture, the rate of cell expansion will start to slow down to the point that it is not advisable, or possible, to culture the cells further.

After injection, the ventral skin of the mice should be monitored every few days. Alopecia can develop as early as 2 weeks after LNC cell suspension injections and normally first occurs at sites other than the injection site. However, initial development of hair loss will more typically take 8-14 weeks (Fig. 4), progressing to near-total hair loss by $\sim$20 weeks after cell transfer. Mice can be reinjected a week after the initial injection with another 10 million cultured LNCs to increase the rate of AA development (unpublished observations).

Extent of alopecia in preclinical trials can be semi-quantified once the stages of onset of lesions are understood. One such grading scheme is illustrated in Figure 5 (Sundberg et al., 2019).

Mice have prolonged telogen (resting) hair cycles, especially as adults. Mild injury, such as wax stripping to remove hair prior to cell injection, will induce initiation of anagen, the active growing phase of the hair cycle (Ito et al., 2020; Sundberg et al., 2012). This method may be utilized if rapid hair regrowth is necessary as part of the subsequent analysis, as with investigations on hair follicle infiltration patterns by immune cells during the early phases of AA pathogenesis, for example. With wax stripping of hair, as opposed to just shaving, hair will begin to regrow at the injection site after ∼1 week. Whether intentional anagen induction could accelerate the onset of overt AA in cell injected mice has not been investigated in any meaningful way.

More recently, studies from Japan have suggested that it may be possible to store fresh lymph node derived cells from spontaneous AA mice in liquid nitrogen, and later use the stored cells in an amplification protocol similar that described here, for subsequent injection into multiple recipients (Hashimoto, Yamada, Sekiguchi, et al., 2021). This approach is currently being validated in other laboratories. The ability to store AA lymph node cells could significantly reduce time, as well as costs, to produce AA-affected mice for sequential studies. Storing cells could also avoid the time and effort needed to maintain a large colony of C3H/HeJ mice to provide spontaneous AA donors.

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Author Contributions

John P. Sundberg: Conceptualization; funding acquisition; investigation; methodology; project administration; resources; supervision; validation; visualization; writing—original draft; writing—review and editing. **Eddy H. C. Wang**: Methodology; visualization; writing—review and editing. **Kevin J. McElwee**: Conceptualization; investigation; methodology; project administration; resources; supervision; validation; visualization; writing—original draft; writing—review and editing.

Conflict of Interest

KJM is a shareholder, consultant, and investigator for Replicel Life Sciences, and director of McElwee Consulting Ltd. The authors state no other conflicts of interest.

Data Availability Statement

No data are presented relative to the protocols described, and therefore there are no data provided to public databases.

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